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USING THE FLOW CYTOMETRY TO QUANTIFY THE *GIARDIA* CYSTS AND CRYPTOSPORIDIUM OOCYSTS IN WATER SAMPLES

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Abstract. The flow cytometry (FC) has been used to detect *Giardia* cysts and *Cryptosporidium* oocysts quantitatively and instantaneously in this study. The experimental results showed that FC is potential to become a more precise method for the detection of *Giardia* and *Cryptosporidium* in water. This study also evaluated the staining efficiencies for three commercial antibodies. After staining *Cryptosporidium* oocysts with direct immunofluorescent antibodies in water samples, two populations were detected in the scatter-plots (FL1 versus SSC) of the FC. The *Cryptosporidium* oocysts and *Giardia* cysts are significantly separated from other particles while stained with direct immunofluorescent antibodies produced by Meridian Diagnostics and Waterborne™ Inc.

Keywords: *Cryptosporidium*, flow cytometry, *Giardia*, protozoan parasites

1. Introduction

The existence of protozoan parasites, *Giardia* cysts and *Cryptosporidium* oocysts, is a common cause of diarrhoea to human (Cook, 1995; Frost *et al.*, 1996). Many surface waters are often contaminated by (oo)cysts from agriculture run-off and sewage effluent because of infected farm stock, wildlife and human being. Both of cysts and oocysts are environmentally robust and may survive in aquatic environments for months. Several waterborne outbreaks of *Giardia* and *Cryptosporidium* to humans have been highly profiled. Many researches have indicated the modern water treatment plants and public swimming pools, which are likely the origins of the outbreaks (Eisenberg *et al.*, 1998). In addition, some useful methods to detect *Giardia* and *Cryptosporidium* in drinking water systems have been suggested so as to prevent future outbreaks from these parasites (Perz *et al.*, 1998; Teunis *et al.*, 1997).

Information collection rule (ICR) method and Method 1623 are commonly used to detect *Giardia* and *Cryptosporidium* in the water samples. The ICR method is considered to be inefficient and labor-intensive. The Method 1623 was also criticized by its high cost in its filtration and separation steps. Moreover, both

methods use the fluorescent microscope to identify (oo)cysts. The microscopic examination is usually hampered by the presence of debris, algae, and autofluorescing particles in water samples. Therefore, there is a demand for the highly trained microscopists for protozoa identification and confirmation (Clancy *et al.*, 1994).

The flow cytometry has been suggested to be a more effective method than fluorescence microscopy for the purpose of quantifying *Giardia* cysts and *Cryptosporidium* oocysts. In addition, the flow cytometry offers the prospect of real-time microbial analysis of microorganism, therefore, it has the potential to be a more precise method for the detection of *Giardia* and *Cryptosporidium* (Arrowood *et al.*, 1995; Vesey *et al.*, 1997; Ferrari *et al.*, 1999). Flow cytometry with fluorescence-activated cell sorting is capable of discriminating and separating Mab-stained (oo)cysts from the other debris. One of the major concerns for water treatment plant using the flow cytometry is the cost of testing. The cost of flow cytometry excluding sorting is estimated to be 3/5 price less compared with the flow cytometry with sorting. If the flow cytometry without sorting system can work well for determing both parasites in water samples, it will be more acceptable equipment for water treatment plants. In this study, we evaluated that the flow cytometry without sorting can be used to detect the oocysts, which is stained by immunofluorescent antibody.

2. Materials and Methods

2.1. Preparation of *Giardia* cysts and *Cryptosporidium* oocysts

Cysts and oocysts used in this study were obtained from the Waterborne, Inc. (Louisiana, U.S.A). These protozoan parasites were stored in 0.01 M phosphate bufferted saline (150 mM NaCl, 15 mM KH_2PO_4, 20 mM Na_2HPO_4, 27 mM KCl, pH 7.4 $\pm$ 0.2) (PBS) at a concentration of 1×10^6 (oo)cysts/mL. The (oo)cysts were diluted with a 0.01 M PBS to desired concentrations as stock solutions. The stock solutions were all stored at 4 °C and were thoroughly mixed before each use. The numbers of cysts and oocysts in the stock solutions were counted using the immunofluorescence assay technique. In order to accurately quantify the cysts and oocysts, samples were mixed thoroughly and pipetted directly from the stock preparation vial and then onto the glass slides (Dynal® Spot-On). It is noted that the glass slides should be stained with fluorescent-labeled antibodies, and pre-washed with 0.1% PBS solution before counting.

2.2. Staining water samples

Three kinds of immunofluorescent antibodies, Direct Immunofluorescent Assay (Cat. No. 250 050, Meridian Diagnostics, Inc., Cincinnati, Ohio), Crypt-a-Glo™ (A400, Waterborne™, Inc., New Orleans, LA, U.S.A.), Giardi-a-Glo™ (A300, Waterborne™, Inc., New Orleans, LA, U.S.A.) and Hydrofluor™ Combo (Cat.

No. 7 080 000, Strategic Diagnostics Inc., Newark, DE, U.S.A.) were used in this study.

The stock solution of *Giardia* and *Cryptosporidium* was diluted to 10 000 cysts and 10 000 oocysts per mL^{-1}, respectively. An aliquot of 100 μL of diluted stock solution was taken into vessels and 90 μL of antibody was added to the dilute stock solution. After incubation at room temperature (about 25 °C) for 30 min, PBS buffer was added to a total volumes of 2100 μL in the sample, and centrifuged at 3500 rpm for 25 min at room temperature. The supernatant was siphoned down to 200 μL, the PBS was added to 700 μL for the final volumes. Samples were mixed by vortexing for 5 s and analyzed by flow cytometry.

2.3. Flow cytometry

The flow cytometry used in this study is Becton Dickinson FACScan Flow Cytometer (Becton Dickinson, NJ, U.S.A.). Samples were collected as sheath fluid and the sample flow rate was set on the "Lo" speed. The data acquisition dot plot parameters used during the analysis were forward angle light scatter (FSC), side angle light scatter (SSC), and FITC fluorescence (FL1). Instrument controls (photomultiplier tube voltage, amplifier gains) follow the standard performance procedures.

2.4. Data analysis

To ensure reproducible results, all samples were delivered to the sample line of the flow cytometry set to a designated condition. Three tubes of the same samples were read to the flow cytometry repeatedly to obtain a consistent output data. The data analysis was carried out with CellQuest software obtained from Becton-Dickinson Biosciences. The analysis program WinMDI was used for all data presentation of CellQuest data files.

3. Results and Discussion

3.1. The detection of *Cryptosporidium* oocysts

Both stained and nonstained *Cryptosporidium* oocysts were observed by flow cytometry scatter. Figures 1 and 2 show the *Cryptosporidium* oocysts scatter-plots representing FSC (x-axis) versus SSC (y-axis), and FL1 (x-axis) versus SSC (y-axis), respectively. In Figures 1 and 2, (a) represents the scatter-plots for *Cryptosporidium* oocysts without staining; (b) is the scatter-plots for *Cryptosporidium* oocysts stained with direct immunofluorescent antibody produced by Waterborne^{TM} Inc.; (c) is the scatter-plots for *Cryptosporidium* oocysts stained with direct immunofluorescent antibody produced by Meridian Diagnostic Inc.; (d) is the scatter-plots

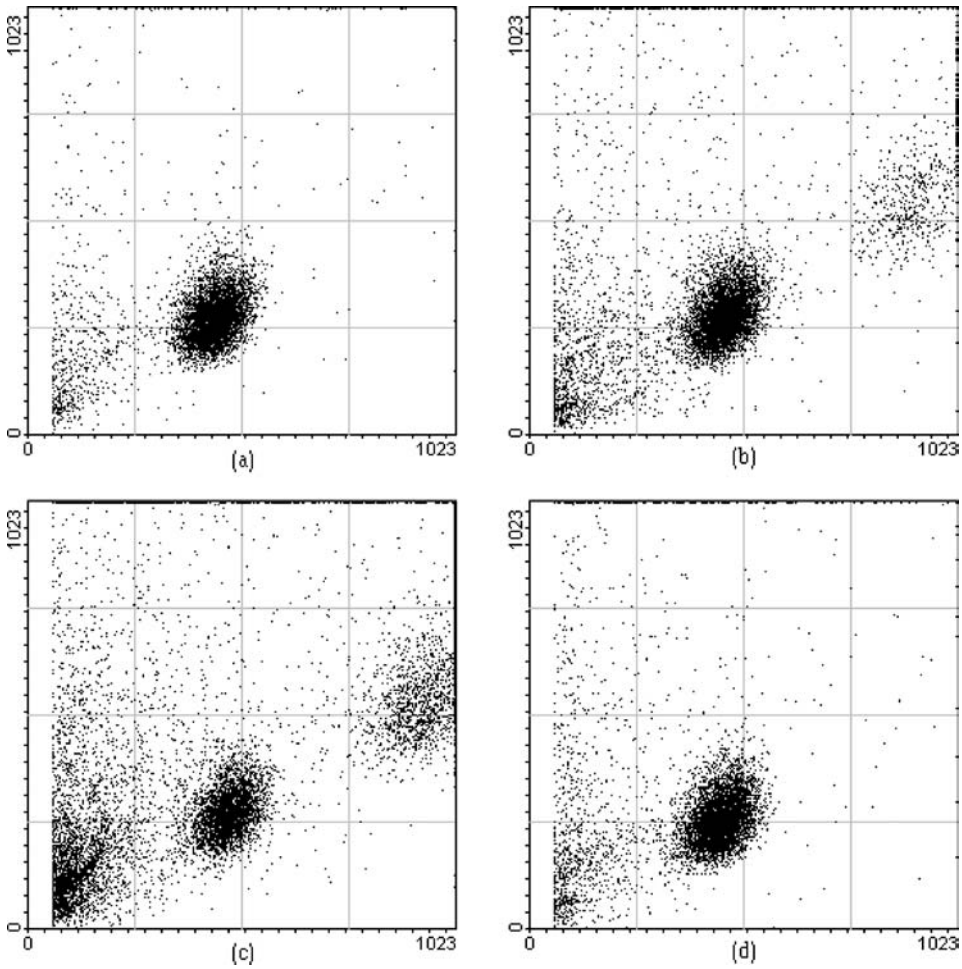

Figure 1. The scatter-plots for *Cryptosporidium* oocysts representing FSC (x-axis) versus SSC (y-axis). (a): The scatter-plot for oocysts without staining. (b): The scatter-plot for oocysts stained with direct immunofluorescent antibody produced by Waterborne^{TM} Inc. (c): The scatter-plot for oocysts stained with direct immunofluorescent antibody produced by Meridian Diagnostic Inc. (d): The scatter-plot for oocysts stained with the indirect immunofluorescent antibody produced by Strategic Diagnostic Inc.

for *Cryptosporidium* oocysts stained with the indirect immunofluorescent antibody produced by Strategic Diagnostic Inc. As depicted in the scatter-plots of Figures 1a and 1b, the *Cryptosporidium* oocysts formed a concentrated population. Comparing the plots of *Cryptosporidium* oocysts staining and nonstaining in Figure 1, the location of oocysts in the plot was not shift. The impurities of signal were observed in the Figures 1b and 1c. The results showed here may be due to insufficient washing steps while staining. While comparing the showing location of oocysts population between Figures 2a and 2c, the single population was divided into two populations.

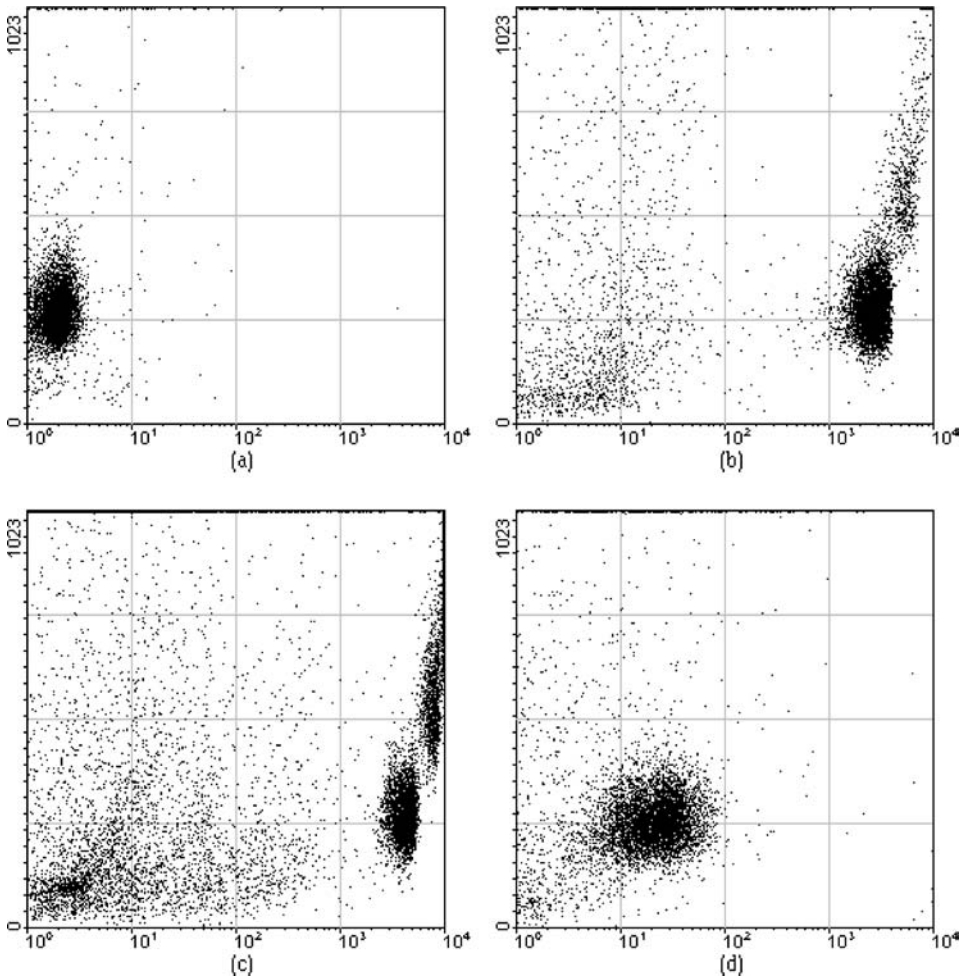

Figure 2. The scatter-plots for *Cryptosporidium* oocysts representing FL1 (x-axis) versus SSC (y-axis). (a): The scatter-plot for oocysts without staining. (b): The scatter-plot for oocysts stained with direct immunofluorescent antibody produced by Waterborne™ Inc. (c): The scatter-plot for oocysts stained with direct immunofluorescent antibody produced by Meridian Diagnostic Inc. (d): The scatter-plot for oocysts stained with the indirect immunofluorescent antibody produced by Strategic Diagnostic Inc.

The large populations in Figures 2b and 2c have a same SSC value with the population on Figure 2a. However, the strength of fluorescence (FL1) on Figures 2b and 2c are 1000 times higher than Figure 2a. The strength of fluorescence on Figure 2d is only 10 times higher than Figure 2a. The results indicated that the efficiency of immuno-staining on Figure 2d is not enough, and the staining procedure is not able to distinguish *Cryptosporidium* oocysts between other debris effectively in the scatter-plot. It is postulated that reducing the washing frequency may increase the fluorescence strength for the indirect immunofluorescent antibodies. The lesser

populations on Figures 2b and 2c have higher FL1 and SSC levels than the large population. Therefore, it is suggested that the oocysts was coagulated in the staining procedures. The intense detergent may be required to separate the coagulated oocysts in the washing steps.

3.2. THE DETECTION OF *GIARDIA* CYSTS

The stained and nonstained *Giardia* cysts with three immunofluorescent antibodies were observed by the FACScan flow cytometer. Figures 3 and 4 show the

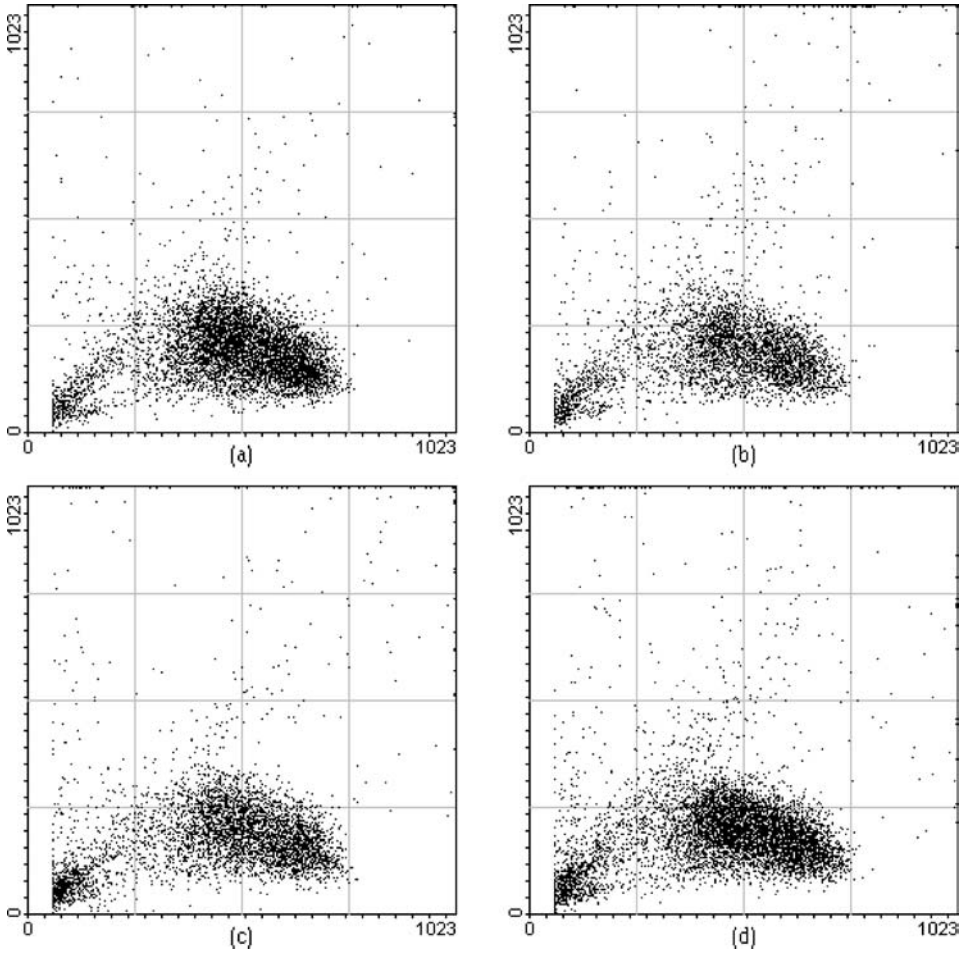

Figure 3. The scatter-plots for *Giardia* cysts representing FSC (x-axis) versus SSC (y-axis). (a): The scatter-plot for cysts without staining. (b): The scatter-plot for cysts stained with direct immunofluorescent antibody produced by Waterborne™ Inc. (c): The scatter-plot for cysts stained with direct immunofluorescent antibody produced by Meridian Diagnostic Inc. (d): The scatter-plot for cysts stained with the indirect immunofluorescent antibody produced by Strategic Diagnostic Inc.

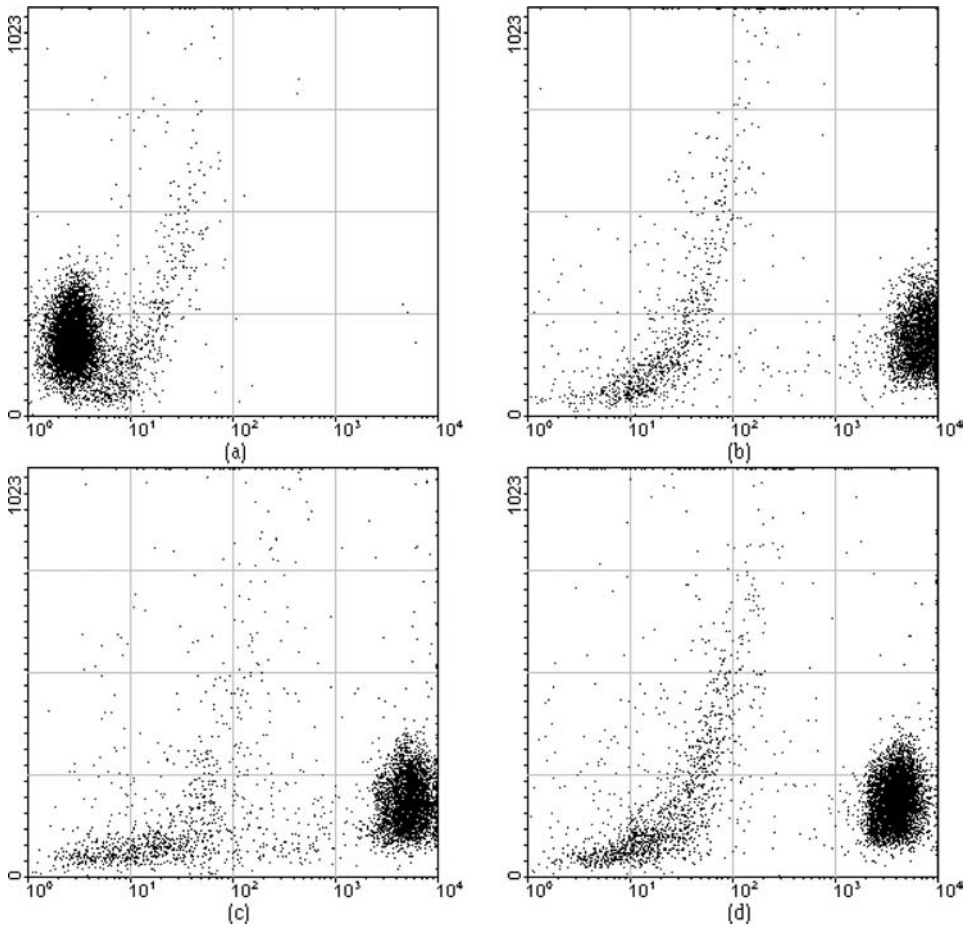

Figure 4. The scatter-plots for *Giardia* cysts representing FL1 (x-axis) versus SSC (y-axis). (a): The scatter-plot for cysts without staining. (b): The scatter-plot for cysts stained with direct immunofluorescent antibody produced by Waterborne^{TM} Inc. (c): The scatter-plot for cysts stained with direct immunofluorescent antibody produced by Meridian Diagnostic Inc. (d): The scatter-plot for cysts stained with the indirect immunofluorescent antibody produced by Strategic Diagnostic Inc.

scatter-plots representing FSC (x-axis) versus SSC (y-axis), and FL1 (x-axis) versus SSC (y-axis), respectively. In Figure 3, the populations of cysts were observed on the same location on (a), (b), (c) and (d). However, the sites for cysts was much more scatter than with oocysts in Figure 1. The shape of cysts for the *Giardia* cysts is elliptic-like, while the oocysts is spherical. When the cysts flow through the detecting tube, the entering position of cysts may influent the detecting values of FSC. In Figure 4, the stained cysts show a high fluorescence strength. The population of cysts was completely separated from background debris. No matter direct or indirect immunofluorescent antibody show extreme staining results for cysts.

4. Conclusion

While spiking the *Cryptosporidium* oocysts and *Giardia* cysts to the water sample and staining (oo)cysts with direct immunofluorescence antibody, two high fluorescence strength populations were detected in their flow cytometry scatter-plots. It can be concluded that direct immunofluorescent antibodies produced by the Meridian Diagnostics and the Waterborne^{TM} Inc., respectively, can be potentially used in flow cytometry for routing monitoring the (oo)cysts in water.

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